

5-Hydroxytryptamine Stimulates Net Ca^{2+} Flux in the Ventricular Muscle of a Mollusc (*Busycon canaliculatum*) During Cardioexcitation

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Abstract. Noninvasive, self-referencing calcium (Ca^{2+}) electrodes were used to study the mechanisms by which 5-hydroxytryptamine (5-HT) affects net Ca^{2+} flux across the sarcolemma of myocytes from ventricular trabeculae (from a marine gastropod, *Busycon canaliculatum*). Treatment of isolated trabeculae with 5-HT causes a net Ca^{2+} efflux, which is 30% blocked by verapamil. These findings suggest that the efflux is in part the result of a previous Ca^{2+} influx through L-type Ca^{2+} channels and is due to a rapid Ca^{2+} extrusion mechanism inherent to the sarcolemma of these myocytes. 5-HT-induced net Ca^{2+} efflux is also reduced by about 40% by treatment with a sodium (Na^+)-free, lithium (Li^+)-substituted saline, which shuts down the Na-Ca exchanger during Ca^{2+} extrusion. Cyclopiazonic acid (CPA), an inhibitor of the sarcoplasmic reticulum (SR) Ca^{2+} ATPase, almost completely abolishes the 5-HT-induced net Ca^{2+} efflux, suggesting that the SR rather than the extracellular pool is the primary Ca^{2+} reservoir serving 5-HT-induced excitation.

Introduction

The rhythmicity of molluscan cardiac muscle is regulated by numerous neurotransmitters and neuromodulatory agents, the most widely studied of these being the biogenic amine 5-hydroxytryptamine (5-HT), the tetrapeptide FMRFamide, and acetylcholine (ACh). Pharmacological application of 5-HT onto the isolated hearts of the gastropods *Busycon canaliculatum* (Hill, 1958; Huddart and Hill, 1996), *Lymnaea stagnalis* (Buckett *et al.*, 1990), and *Aplysia californica* (Liebeswar *et al.*, 1975) causes a dose-dependent increase in inotropic and chronotropic activity

with a primary effect on long-duration chronotropic regulation. Underlying this potentiation in cardiac rhythmicity is an increase in the amplitude and frequency of cardiac action potentials as well as the coupled systolic force (Hill *et al.*, 1992). The combined anatomical, pharmacological, and physiological studies on the gastropods *Aplysia*, *Busycon*, and *Lymnaea* have provided overwhelming evidence that 5-HT acts as a cardioexcitatory neurotransmitter in this class of mollusc.

$^{45}\text{Ca}^{2+}$ efflux studies have been conducted on a variety of molluscan cardiac and smooth muscles to determine how Ca^{2+} ions may be mobilized by 5-HT during the process of contraction or relaxation (Bloomquist and Curtis, 1972, 1975; Koch and Greenberg, 1981; Sawada *et al.*, 1984; Ishii *et al.*, 1989). The present study uses a newer technique to study Ca^{2+} flux, noninvasive self-referencing Ca^{2+} electrodes, to determine possible mechanisms by which 5-HT affects trans-sarcolemmal net Ca^{2+} flux in the *Busycon* ventricle as a clue to understanding its role during the excitation-contraction (E-C) coupling in the gastropod heart. An earlier study focused on the action of FMRFamide on net Ca^{2+} flux in the ventricle of *Busycon canaliculatum* (Devlin, 1997), so we may be able to compare mechanisms working at putative Ca^{2+} pools accessed by 5-HT and FMRFamide to achieve the same end—enhanced cardiac performance.

Materials and Methods

Specimens of *Busycon canaliculatum*, the channeled whelk, were obtained on the day of each experiment from the Marine Resources Center of the Marine Biological Laboratory (MBL), Woods Hole, Massachusetts. All experiments were conducted at the National Institutes of Health BioCurrents Research Center located at the MBL.

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Preparation

The shell was completely cut away from the animal with bone forceps, exposing the heart and its enveloping opaque pericardium. The pericardium was opened, and the ventricle was removed by severing its connections to the aorta and atrium. The ventricle was opened and pinned down in a dish of natural seawater to expose the inner latticework of trabeculae. An individual trabecula was isolated with fine scissors from the inner wall of the ventricle and secured with minuten pins to a Sylgard well in a small recording chamber. The trabecula was bathed in a nominally Ca^{2+} -free artificial seawater (ASW) prepared according to the MBL formula (423 mM Na^+ , 9.7 mM K^+ , 9.9 mM Ca^{2+} , 51.2 mM Mg^{2+} , 538.6 mM Cl^- , 27 mM SO_4^{-2} , 2.3 mM HCO_3^{-}). Magnesium was used to replace the 9.9 mM Ca^{2+} omitted from the Ca^{2+} -free ASW. The nominal (or background) amounts of Ca^{2+} ions still present in the Ca^{2+} -free ASW were typically 100 micromolar (μM) or less and were monitored continuously with a Ca^{2+} electrode throughout the experiment.

Ion flux measurement

The self-referencing ion electrode technique is used to detect net ion flux generated from a biological source, in this case, the sarcolemma of myocytes from a *Busycon* trabecula. The net ion flux across the membrane is the sum of both inward and outward ion movements. In the present experiments, electrodes loaded with a Ca^{2+} -specific ionophore (Fluka Chemika Ca^{2+} ionophore—cocktail A with the neutral carrier ETH 1001) were used to detect net Ca^{2+} flux (in $\text{pmol cm}^{-2} \text{ s}^{-1}$) across the sarcolemma of the myocytes. The electrode was programmed with PC-based software (Ionprobe) to oscillate with an excursion of 10 μm and a slow frequency of 0.3 Hz; this minimized mixing of the bathing saline. The difference in voltage (μV) between the two ends of the excursion was measured and could be taken as corresponding to a Ca^{2+} concentration gradient, since the excursion was constant.

To construct the electrodes, borosilicate micropipettes were pulled from 1.5-mm-diameter glass capillaries (World Precision Instruments, Inc.), then back-filled with 100 mM CaCl_2 in 0.1% agar. The pipette was front-filled with calcium ionophore—cocktail A containing neutral carrier ETH 1001 (Fluka Chemika) to produce an ionophore column of 40 μm . A Ag/AgCl wire inserted into the back of the micropipette served as the coupling to the headstage. To calibrate the electrode prior to the experiment, the electrode was tested in 0.1 mM, 1 mM, and 10 mM Ca^{2+} solutions to check its Nernstian properties—that is, an approximate 28 mV difference per decade change in Ca^{2+} concentration. The return electrode was a Ag/AgCl wire inserted into a glass capillary containing 3 M KCl agar.

The electrode oscillated at right angles to the long axis of

the trabecula and was positioned at two distances from the muscle surface. One electrode position was 500 μm away from the muscle surface. At this relatively great distance, Ca^{2+} flux from the ion source (*i.e.*, the muscle) cannot be detected. Thus, only the control, background levels of Ca^{2+} in the saline are recorded at this position. The second electrode position was only 5 μm from the muscle surface, so net Ca^{2+} flux could be measured directly at the sarcolemma. From the background level of Ca^{2+} ions in the bathing saline, the Ca^{2+} ion concentration gradient at the muscle surface, and the diffusion constant for Ca^{2+} ions, net Ca^{2+} ion flux was calculated using a modification of the Fick equation. The mathematical formulas used in the conversion of voltage to flux units, and other technical aspects and applications of the self-referencing electrode technique, are described by Smith *et al.* (1999).

Pharmacological agents

The neurotransmitter, 5-HT, and the L-type channel blocker, verapamil, were obtained from Sigma Chemical Company (St. Louis, MO). Verapamil was selected over other Ca^{2+} blockers because of its consistent antagonistic action on other invertebrate cardiac and smooth muscle types (Devlin 1993a, b, 1997, Devlin and Smith, 1996). Diltiazem or nifedipine, which often act as agonists in molluscan muscle preparations, were not used in this study. The Ca^{2+} channel agonist, Bay K 8644, and the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor, cyclopiazonic acid (CPA), were obtained from Research Biochemicals International (Natick, MA). At the beginning of each experiment each drug was diluted in the nominal Ca^{2+} -free ASW to achieve the designated experimental concentration.

Experimental protocol

The following general protocol was used throughout the present experiments. An initial net Ca^{2+} efflux generated by 10^{-7} M 5-HT (the control) was recorded during a 3-min treatment; this net Ca^{2+} efflux immediately returns to basal levels when the 5-HT is washed from the trabecula by flushing with ASW for 20 to 30 min. Next, the trabecula was pretreated for 10 min with verapamil or Bay K 8644 (10^{-5} M) or other inhibitors (CPA or Li^+ ASW). 5-HT (10^{-7} M) dissolved in solutions of verapamil or Bay K 8644 (10^{-5} M) or other inhibitors (CPA or Li^+ ASW) was reapplied to the trabecula for a 3-min treatment period. The control, the initial response to 5-HT alone, was then compared to the response to 5-HT in the presence of the agonist, antagonist, or inhibitor. A *t*-test analysis was then performed.

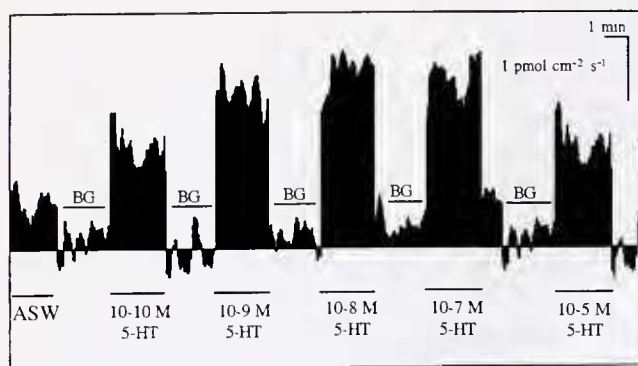


Figure 1. An experimental record showing a concentration-dependent effect of 5-HT on net Ca^{2+} efflux (in $\text{pmol cm}^{-2} \text{s}^{-1}$). ASW = spontaneous net Ca^{2+} flux measurement taken when the electrode was placed directly at the ion source, the trabecula, at a distance of less than $5 \mu\text{m}$ during treatment with an artificial Ca^{2+} -free seawater (ASW). 5-HT (10^{-10}M to 10^{-5}M) = net Ca^{2+} efflux measurement taken when the electrode was still placed directly at the trabecula at a distance of less than $5 \mu\text{m}$ during treatment with a range of 5-HT concentrations. Maximum net efflux was recorded between 10^{-8}M and 10^{-7}M 5-HT. Background (BG) = small oscillations around the baseline that were recorded when the electrode was placed $500 \mu\text{m}$ from the ion source, the trabecula.

Results

With the electrode placed $5 \mu\text{m}$ from the muscle surface, a basal net Ca^{2+} efflux of $1.21 \pm 0.42 \text{ pmol cm}^{-2} \text{s}^{-1}$ ($n = 10$) is recorded from the trabecula at rest in Ca^{2+} -free ASW. A range of 5-HT concentrations (10^{-12}M to 10^{-5}M) was then tested. Above a threshold of about 10^{-10}M , all concentrations of 5-HT enhance basal net Ca^{2+} efflux. The maximal efflux is induced at 5-HT between 10^{-8} – 10^{-7}M ; above 10^{-7}M , the net Ca^{2+} efflux is actually smaller than those induced by lower 5-HT concentrations (Fig. 1). Thus, the 5-HT receptor was desensitized by exposures to higher doses of its ligand. Because the effect of 10^{-7}M 5-HT does not desensitize the receptor and is completely reversible, it was chosen as the concentration to be challenged by various Ca^{2+} channel antagonists or agonists. A stable net Ca^{2+} efflux induced by 10^{-7}M 5-HT is on the order of $2.63 \pm 1.01 \text{ pmol cm}^{-2} \text{s}^{-1}$ ($n = 8$).

Effect of Ca^{2+} -channel antagonists or agonists on 5-HT-induced net Ca^{2+} efflux

To test the hypothesis that the Ca^{2+} ions mobilized by 5-HT during E-C coupling are from the extracellular saline, I studied the effects of two L-type Ca^{2+} channel drugs, an antagonist (verapamil) and an agonist (Bay K 8644), on the 5-HT response. In concentration-response experiments conducted with verapamil or Bay K 8644 alone, neither drug has a significant effect on net Ca^{2+} flux over the concentration range tested (10^{-12} to 10^{-5}M) (not shown). However, verapamil inhibits the action of 5-HT, reducing the response to $67\% \pm 16\%$ ($n = 3$, $P < 0.025$) of the control

(5-HT-induced net efflux measured prior to verapamil treatment) (Fig. 2). After verapamil was washed from the preparation, subsequent exposures to 5-HT are often enhanced. Bay K 8644 has no significant effect on the 5-HT response.

The effect of a Na^+ -free, Li^+ -substituted ASW on 5-HT responses

To determine whether the net Ca^{2+} efflux was mediated by the Na^+ - Ca^{2+} exchanger, sodium (Na^+) ions were omitted from the bathing saline and replaced instead with an equivalent concentration (423mM) of lithium (Li^+) ions; the rationale is that Li^+ transverse the Na^+ channel but cannot be substituted for Na^+ in the Na^+ - Ca^{2+} exchanger (Lipp and Niggli, 1994). The 5-HT-induced net Ca^{2+} efflux is reduced by the presence of Li^+ ions to $57\% \pm 9\%$ ($n = 3$, $P < 0.01$) that of control (the 5-HT response in normal ASW) (Fig. 3), implicating the Na^+ - Ca^{2+} exchanger as a mechanism of Ca^{2+} extrusion during excitation by 5-HT. Li^+ treatment alone reduced the basal net Ca^{2+} efflux to $69\% \pm 11\%$ ($n = 3$, $P < 0.01$).

The effect of cyclopiazonic acid (CPA) on 5-HT responses

Figure 4 shows the effect of SERCA inhibitor CPA (10^{-5}M) on 5-HT-induced net Ca^{2+} efflux. The 5-HT response is

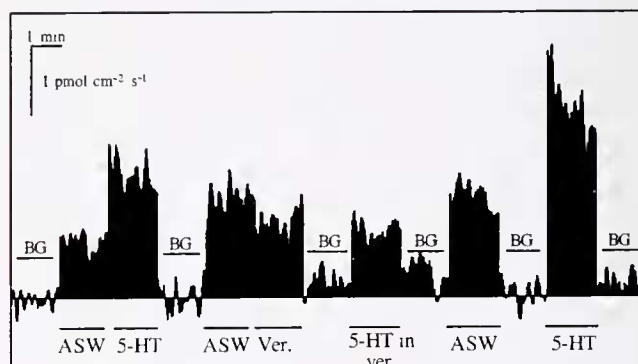


Figure 2. An experimental record showing that the 5-HT-induced net Ca^{2+} efflux signal (in $\text{pmol cm}^{-2} \text{s}^{-1}$) is inhibited by the L-type channel antagonist verapamil (10^{-5}M). ASW = spontaneous net Ca^{2+} efflux measurement taken when the electrode was placed directly at the ion source, the trabecula, at a distance of less than $5 \mu\text{m}$. This signal was recorded prior to any drug treatment when the trabecula was bathed only in an artificial Ca^{2+} -free seawater (ASW). 5-HT (1st) = net Ca^{2+} efflux measurement taken when the electrode was placed directly at the trabecula during treatment with 10^{-7}M 5-HT. Verapamil = net Ca^{2+} efflux measurement taken when the electrode was placed directly at the trabecula after a 10-min treatment with verapamil (10^{-5}M). 5-HT in ver = net Ca^{2+} efflux measurement taken when the electrode was placed directly at the trabecula when treated with 5-HT (10^{-7}M) in verapamil (10^{-5}M). After verapamil was washed from the muscle, the final response to 5-HT (2nd) was greatly enhanced. All above recordings at the trabecula were taken at distance of $5 \mu\text{m}$ or less. Background (BG) = small oscillations around the baseline that were recorded when the electrode was placed $500 \mu\text{m}$ from the ion source, the trabecula.

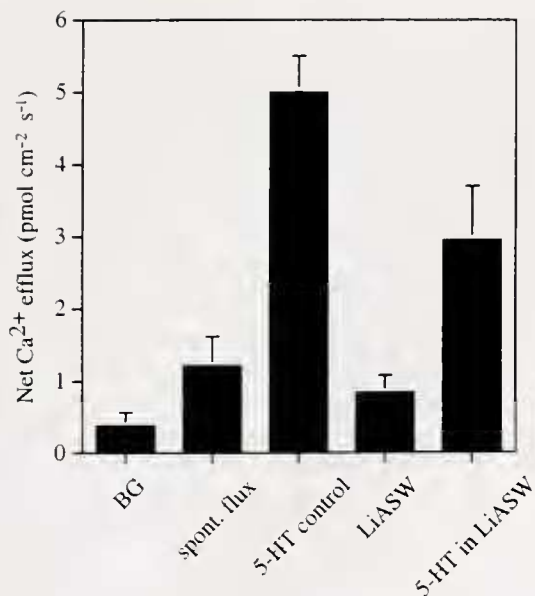


Figure 3. The inhibitory effect of LiASW on 5-HT-induced net Ca^{2+} efflux (in $\text{pmol cm}^{-2} \text{s}^{-1}$). First bar, background (BG): Net Ca^{2+} efflux measurement taken when the electrode was placed 500 μm from the ion source, the trabecula. Second bar, spontaneous flux: Net Ca^{2+} efflux measurement taken when the electrode was placed directly at the ion source, the trabecula, at a distance of less than 5 μm during treatment with an artificial seawater. Third bar, 5-HT control: Net Ca^{2+} efflux measurement taken when the electrode was still placed directly at the trabecula during treatment with 5-HT (10^{-7} M). Fourth bar: After washing the muscle for 30 min with artificial seawater, net Ca^{2+} efflux measurement was taken when the electrode was placed directly at the ion source, the trabecula, at a distance of less than 5 μm during treatment with a Na-free, Li-substituted artificial seawater (LiASW). Fifth bar, 5-HT in LiASW: Net Ca^{2+} efflux measurement taken when the electrode was still placed directly at the muscle during treatment with 5-HT (10^{-7} M) in LiASW. The data are means pooled from three muscles \pm SD.

inhibited by CPA to $18.5\% \pm 10\%$ ($n = 3$, $P < 0.005$) of the control (5-HT-induced flux prior to CPA treatment). CPA (10^{-5} M) alone also reduces basal net efflux to $76\% \pm 11\%$ ($n = 3$, $P < 0.025$) that of the control. These data indicate that excitation by 5-HT relies more on Ca^{2+} ions from the sarcoplasmic reticulum (SR) than does spontaneous myogenicity. The inhibitory effect of CPA on 5-HT responses is completely reversible.

Discussion

The present experiments showed that 5-HT stimulates a net efflux of Ca^{2+} in the ventricular muscle of the whelk *Busycon canaliculatum*. The Ca^{2+} signal is sensitive to partial block by verapamil, which suggests that some of the Ca^{2+} movement is through sarcolemmal L-type Ca^{2+} channels. These L-type channels account for about 20% of the Ca^{2+} mobilized into the internal compartment during E-C coupling and may provide the source for Ca^{2+} -induced Ca^{2+} release from the SR, resulting in increased systolic

force. The remaining 80% of Ca^{2+} ions are probably released from the SR, as shown by experiments with SERCA inhibitor CPA, which almost completely blocks the 5-HT response. Li^+ ions, which act to block the Na-Ca exchange process, partially inhibit the 5-HT response, suggesting some reliance on the Na^+ - Ca^{2+} exchanger during the Ca^{2+} extrusion process.

Verapamil, an L-type Ca^{2+} channel blocker, inhibits 5-HT-induced net Ca^{2+} efflux by about 30% in myocytes of the *Busycon* trabeculae. Similarly, in both gastropod (Hud-dart and Hill, 1996) and bivalve ventricles (Devlin, 1993)—where 5-HT enhances both inotropic and chronotropic responses—the effect of 5-HT is dependent on the movement of extracellular Ca^{2+} ions through an L-type channel, since 5-HT responses are blocked by verapamil (Devlin, 1993b). Although verapamil has no significant effect on basal net Ca^{2+} efflux in the *Busycon* ventricle, it does inhibit the 5-HT response, suggesting a use-dependent mechanism of block. This same use-dependent block by verapamil also occurs in many other invertebrate and vertebrate muscle preparations that were either chemically or electrically stimulated (Lee and Tsien, 1983; Zahradnik and Zachar, 1983; Vaghy *et al.*, 1988; Nanasi *et al.*, 1990).

Whereas verapamil inhibits the 5-HT-induced net Ca^{2+} efflux from the *Busycon* cardiac myocytes by about 30%, it

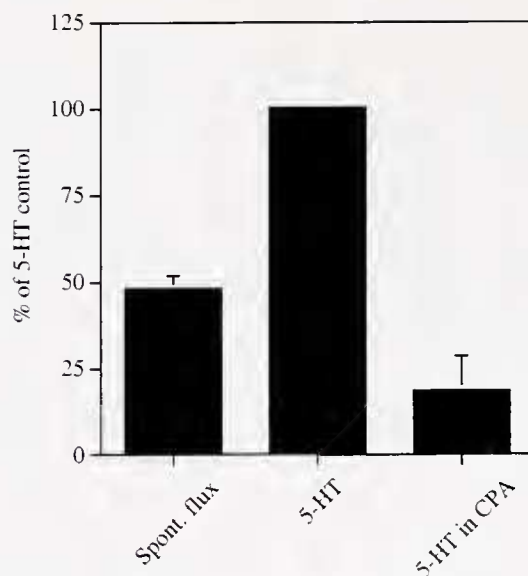


Figure 4. The inhibitory effect of SERCA inhibitor, cyclopiazonic acid (CPA) on 5-HT-induced net Ca^{2+} efflux (in $\text{pmol cm}^{-2} \text{s}^{-1}$). First bar, spontaneous flux: Net Ca^{2+} efflux measurement taken when electrode was placed directly at the ion source, the trabecula, at a distance of less than 5 μm during treatment with an artificial seawater. Second bar, 5-HT control: Net Ca^{2+} efflux measurement taken when the electrode was still placed directly at the trabecula during treatment with 5-HT (10^{-7} M). Third bar, 5-HT in CPA: Net Ca^{2+} efflux measurement taken when the electrode was still placed directly at the muscle during treatment with 5-HT (10^{-7} M) in CPA (10^{-5} M). The data are means pooled from three experiments on three ventricles \pm SD.

reduces the FMRFamide response by 60% in the same preparation (Devlin, 1997). Therefore, 5-HT relies less on extracellular Ca^{2+} ions than does FMRFamide during the process of E-C coupling in the *Busycon* ventricle. The results also point to two distinct Ca^{2+} release mechanisms used by these respective neurotransmitters. However, both 5-HT and FMRFamide activate L-type Ca^{2+} channels (whether directly or indirectly remains to be determined) during cardioexcitation, since verapamil partially inhibits net Ca^{2+} efflux induced by either chemical. This finding also indicates that the net Ca^{2+} efflux is in part an effect of an earlier Ca^{2+} influx through L-type channels, possibly carried by the HVA (high-voltage activated) current described by Yeoman *et al.* (1999).

5-HT (from 10^{-9} to 10^{-6} M) depolarizes the heart cells from *Helix pomatia* (Kiss and S.-Rosza, 1978), *Aplysia dactylomela* (Sawada *et al.*, 1984), and *Dolabella auricularia* (Hill, 1974). This is the same concentration range in which 5-HT stimulates a net Ca^{2+} efflux from the ventricular myocytes of *B. canaliculatum*. Two depolarizing Ca^{2+} currents, designated the LVA (low-voltage activated), a T-type current, and the HVA (high-voltage activated), an L-type current, have been identified in the ventricular cells of the gastropod *Lymnaea* (Yeoman *et al.*, 1999). The sequential activation of the LVA and HVA currents provides the mechanism for pacemaking, AP generation, and a Ca^{2+} source for E-C coupling in the gastropod ventricle (Yeoman *et al.*, 1999). Since 5-HT increases the Ca^{2+} -dependent (and a Na^{+} -dependent) component of cardiac APs and coupled systolic force in bivalve ventricles (Devlin, 1993b), potentiation of an HVA-like current may be involved given that the Ca^{2+} -dependent component is sensitive to verapamil, diltiazem, and Bay K 8644. 5-HT also induces a Ca^{2+} current in *Aplysia* RB neurons (Pellmar, 1984) and mammalian neurons (Burnashev, 1998).

The relationship between the gastropod 5-HT receptor and coupled ion channels (such as the L-type channel) remains unclear because the subtypes that mediate excitatory responses in the gastropod heart are still being pharmacologically identified. However, a large body of evidence suggests that the 5-HT receptor from both gastropod (S.-Rosza and Kiss, 1976; Keabian *et al.*, 1979; Mandelbaum *et al.*, 1979; S.-Rosza, 1984; Sawada *et al.*, 1984; Drummond *et al.*, 1985; Huddart and Hill, 1996) and bivalve hearts (Higgins, 1974, 1977; Higgins and Greenberg, 1974; Paciotti and Higgins, 1985) is associated with the adenylate cyclase-cAMP signaling pathway, which when stimulated produces an intracellular rise in cAMP. Higgins and Greenberg (1974) found that cAMP increases the phosphorylation of SR proteins that mediate Ca^{2+} sequestration into microsomes prepared from bivalve hearts. This then decreases the length of the diastolic phase of the cardiac cycle and primes the SR to release more Ca^{2+} ions during the next contraction. cAMP was reported to stimulate Ca^{2+} release from

intracellular stores in molluscan neurons as well (Kononecko *et al.*, 1983). In contrast, FMRFamide does not appear to work through the adenylate cyclase-cAMP signaling pathway in gastropod ventricles (Drummond *et al.*, 1985; Huddart and Hill, 1996) but instead may mediate phosphoinositide hydrolysis, the process that has been implicated in some bivalve molluscs (Bayakly and Deaton, 1992).

Both 5-HT and some cAMP analogs stimulate $^{45}\text{Ca}^{2+}$ efflux from *Aplysia* ventricular myocytes during enhanced chronotropic and inotropic activities (Sawada *et al.*, 1984). In the present experiments, 5-HT also induces a large net Ca^{2+} efflux (on the order of $2.6 \text{ pmol cm}^{-2} \text{ s}^{-1}$) from *Busycon* trabeculae that is recorded even when slow contractions of the trabecula are visible under the microscope. This net Ca^{2+} efflux is sustained over many hours. Sawada *et al.* (1984) also reported that excitatory drugs that stimulate contraction and cause simultaneous $^{45}\text{Ca}^{2+}$ efflux do not deplete cytoplasmic Ca^{2+} in the ventricular myocytes of *Aplysia*. These combined Ca^{2+} efflux data from *Busycon* and *Aplysia* ventricles suggest a substantial internal Ca^{2+} reserve such as the well-developed SR in the gastropod cardiac muscle described by Sanger (1979).

The 5-HT-induced Ca^{2+} efflux recorded from the *Busycon* ventricle is a large, stable signal that immediately returns to control levels upon washing with seawater. In contrast, in bivalve ventricles (from mussel, *Geukensia demissa*) that are excited by 5-HT, a transient $^{45}\text{Ca}^{2+}$ efflux corresponds to the onset of a 5-HT-induced contracture but is not sustained throughout the contracture (Koch and Greenberg, 1981). When 5-HT is washed from the *Geukensia* ventricle, a second large $^{45}\text{Ca}^{2+}$ efflux occurs, during which total tissue Ca^{2+} increases. Sawada *et al.* (1984) similarly noted that Ca^{2+} efflux does not necessarily reflect a reduction in cytoplasmic Ca^{2+} , because internal reserves may be available. A second application of 5-HT to the mussel heart does not induce a subsequent $^{45}\text{Ca}^{2+}$ efflux (Koch and Greenberg, 1981), whereas repeated efflux signals are stimulated by 5-HT in the gastropod heart. These findings reflect the large difference in complexity between the gastropod SR and the bivalve SR as sustainable Ca^{2+} pools.

The SERCA inhibitor, CPA, blocks 5-HT responses by 80% in the *Busycon* heart, suggesting that the SR is the major Ca^{2+} reservoir used during cardioexcitation by 5-HT. CPA acts by inhibiting the binding of Ca^{2+} ions at high-affinity binding sites on the SERCA; this shuts down both Ca^{2+} uptake and subsequent Ca^{2+} release at the SR of skeletal, cardiac (Balke *et al.*, 1994), and smooth muscle (Suzuki *et al.*, 1992). An indirect effect of inhibiting Ca^{2+} sequestration at the SR is a reduction in Ca^{2+} -induced Ca^{2+} release; such a reduction is typically activated by the entry of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels. In short, this would serve to limit the Ca^{2+}

needed to activate the contractile proteins. On the other hand, a reduction in Ca^{2+} -induced Ca^{2+} release also decreases the Ca^{2+} -dependent K^+ current (I_{K-Ca}), thus ultimately prolonging the excitability of vertebrate smooth muscle (Suzuki *et al.*, 1992). How CPA is affecting excitability in the mollusc ventricle warrants further investigation, especially considering its effectiveness in blocking Ca^{2+} efflux and the complete reversibility of its action.

The net Ca^{2+} efflux that was recorded during 5-HT treatment was composed primarily of Ca^{2+} released from the SR and secondarily of Ca^{2+} , from the extracellular fluid, that had previously entered the myocytes *via* an L-type current. This net Ca^{2+} efflux reflects the ability of the sarcolemma to rapidly redistribute Ca^{2+} ions outward, therefore preventing the toxic effects of Ca^{2+} overload, and reestablishing intracellular and extracellular Ca^{2+} gradients before the next successive depolarization and contraction. To determine if the net efflux was in part due to activity of the Na^+ - Ca^{2+} exchanger, a Na^+ -free, Li^+ -substituted saline (Li^+ ASW) was used. The Li^+ ASW reduces the basal net Ca^{2+} efflux during normal, autorhythmic activity of the *Busycon trabeculae* by only 20%–30%, whereas the 5-HT and FMRFamide responses are inhibited during Li^+ treatment by 40% and 73%, respectively (Devlin, 1997). This difference in response suggests a greater reliance on the Na^+ - Ca^{2+} exchanger during chemically mediated excitation than during spontaneous myogenic activity. These Li^+ substitution experiments on the gastropod trabeculae also show that Na^+ ions are a necessary stimulus for Ca^{2+} mobilization through voltage-gate channels or from an intracellular pool, perhaps during Na^+ -induced Ca^{2+} release (Lipp and Niggli, 1994), and that they are also involved in the process of Ca^{2+} extrusion. The data from the present study are in agreement with studies on the Na^+ - Ca^{2+} exchanger in other myocytes where the removal of extracellular Na^+ reduces Ca^{2+} efflux in guinea atrial cells (Reuter and Seitz, 1968) and in internally dialyzed myocytes (Miura and Kimura, 1989).

Acknowledgments

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Literature Cited

- Balke, C. W., T. M. Egan, and W. G. Wier. 1994. Processes that remove calcium from the cytoplasm during excitation-contraction coupling in intact rat heart cells. *J. Physiol.* **474**: 447–462.
- Bayakly, N. A., and L. E. Deaton. 1992. The effects of FMRFamide, 5-hydroxytryptamine and phorbol esters on the heart of the mussel *Geukensia demissa*. *J. Comp. Physiol. B.* **162**: 463–468.
- Bloomquist, E., and B. A. Curtis. 1972. The action of calcium-45 efflux from the anterior byssal retractor muscle of *Mytilus edulis*. *J. Gen. Physiol.* **59**: 476–485.
- Bloomquist, E., and B. A. Curtis. 1975. ^{45}Ca efflux from anterior byssal retractor muscle in phasic and catch contraction. *Am. J. Physiol.* **229**: 1237–1243.
- Buckett, K. J., G. J. Dockray, N. N. Osborne, and P. R. Benjamin. 1990. Pharmacology of the myogenic heart of the pond snail *Lymnaea stagnalis*. *J. Neurophysiol.* **63**: 1413–1425.
- Burnashev, N. 1998. Calcium permeability of ligand-gated channels. *Cell Calcium* **24**: 325–332.
- Devlin, C. L. 1993a. Acetylcholine-induced contractions in a holothurian (*Isostichopus badionotus*) smooth muscle are blocked by the calcium antagonists, diltiazem and verapamil. *Comp. Biochem. Physiol.* **106C**: 573–577.
- Devlin, C. L. 1993b. An analysis of control of the ventricle of the mollusc *Mercenaria mercenaria*. II. Ionic mechanisms involved in excitation by 5-hydroxytryptamine. *J. Exp. Biol.* **179**: 63–75.
- Devlin, C. L. 1997. A vibrating Ca^{2+} -selective electrode measures Ca^{2+} flux induced by the neuropeptide FMRFamide in a gastropod ventricle. *Comp. Biochem. Physiol.* **116A**: 93–100.
- Devlin, C. L., and P. J. S. Smith. 1996. A non-invasive vibrating calcium-selective electrode measures acetylcholine-induced calcium flux across the sarcolemma of a smooth muscle. *J. Comp. Physiol. B* **166**: 270–277.
- Drummond, G. I., S. Wernham, and K. Lukowiak. 1985. Stimulation of adenylate cyclase in the heart of *Aplysia californica* by biogenic amines. *Comp. Biochem. Physiol. C.* **80**: 129–133.
- Higgins, W. J. 1974. Intracellular action of 5-hydroxytryptamine on the bivalve myocardium. I. Adenylate and guanylate cyclases. *J. Exp. Biol.* **190**: 99–110.
- Higgins, W. J. 1977. 5-hydroxytryptamine-induced tachyphylaxis of the molluscan heart and concomitant desensitization of adenylate cyclase. *J. Cyclic Nucleotide Res.* **3**: 293–302.
- Higgins, W. J., and M. J. Greenberg. 1974. Intracellular action of 5-hydroxytryptamine on the bivalve myocardium. II. Cyclic nucleotide-dependent protein kinases and microsomal calcium uptake. *J. Exp. Zool.* **190**: 305–316.
- Hill, R. B. 1958. The effects of certain neurohumors and of other drugs on the ventricle and radula protractor of *Busycon canaliculatum* and on the ventricle of *Strombus gigas*. *Biol. Bull.* **115**: 471–482.
- Hill, R. B. 1974. Effects of 5-hydroxytryptamine on action potentials and on contractile force in the ventricle of *Dolabella auricularia*. *J. Exp. Biol.* **61**: 529–539.
- Hill, R. B., H. Huddart, and C. L. Devlin. 1992. Activation of a molluscan heart. Pp. 149–165 in *Phylogenetic Models in Functional Coupling of the CNS and the Cardiovascular System*, R. B. Hill and K. Kuwasawa, eds. S. Karger Medical and Scientific Publishers, Basel, Switzerland.
- Huddart, H., and R. B. Hill. 1996. Modulatory mechanisms in the isolated internally perfused ventricle of the whelk, *Busycon canaliculatum*. *Gen. Pharmacol.* **27**: 809–818.
- Ishii, N., A. W. M. Simpson, and C. C. Ashley. 1989. Effects of 5-hydroxytryptamine (serotonin) and forskolin on intracellular free calcium in isolated and fura-2 loaded smooth-muscle cells from the anterior byssal retractor (catch) muscle of *Mytilus edulis*. *Pflugers Arch.* **414**: 162–170.
- Kebabian, P. R., J. W. Kebabian, and D. O. Carpenter. 1979. Regulation of cyclic AMP in heart and gill of *Aplysia* by the putative neurotransmitters dopamine and serotonin. *Life Sci.* **24**: 1757–1764.
- Kiss, T., and K. S. Rozsa. 1978. Pharmacological properties of 5-HT receptors of the *Helix pomatia* L. (Gastropoda) heart muscle cells. *Comp. Biochem. Physiol. C.* **61**: 41–46.
- Koch, R. A., and M. J. Greenberg. 1981. Calcium fluxes accompanying

- the action of 5-hydroxytryptamine on mussel hearts. *Comp. Biochem. Physiol. C* **70**: 229-239.
- Kononecko, N. I., P. G. Kostyuk, and A. D. Sherbatko. 1983.** The effect of intracellular cAMP injections on stationary membrane conductance and voltage and time dependent ionic currents in identified snail neurons. *Brain Res.* **268**: 321-338.
- Lee, K. S., and R. W. Tsien. 1983.** Mechanism of calcium channel blockade by verapamil, D600, diltiazem and nitrendipine in single dialyzed heart cells. *Nature* **302**: 790-794.
- Liebeswar, G., J. E. Goldman, J. Koester, and E. Mayeri. 1975.** Neural control of the circulation in *Aplysia*. III. Neurotransmitters. *J. Neurophysiol.* **38**: 767-779.
- Lipp, P., and E. Niggli. 1994.** Sodium current-induced calcium signals in isolated guinea-pig ventricular myocytes. *J. Physiol.* **474**: 439-446.
- Mandelbaum, D. E., J. Koester, M. Schonberg, and K. R. Weiss. 1979.** Cyclic AMP mediation of the excitatory effect of serotonin in the heart of *Aplysia*. *Brain Res.* **177**: 388-394.
- Miura, Y., and J. Kimura. 1989.** Dependence on internal Ca and Na and competitive binding of external Na and Ca. *J. Gen. Physiol.* **93**: 1129-1145.
- Nanasi, P. P., A. Varro, D. A. Lathrop, and M. Danko. 1990.** Use-dependent action of antiarrhythmic drugs in frog skeletal muscle and canine cardiac Purkinje fiber. *Gen. Pharmacol.* **21**: 747-751.
- Paciotti, G. F., and W. J. Higgins. 1985.** Potentiation of the 5-hydroxytryptamine-induced increases in myocardial contractility in *Mercenaria mercenaria* ventricle by forskolin. *Comp. Biochem. Physiol. C* **80**: 325-329.
- Pellmar, T. C. 1984.** Enhancement of inward current by serotonin in neurons of *Aplysia*. *J. Neurophysiol.* **15**: 13-25.
- Reuter, H., and N. Seitz. 1968.** The dependence of calcium efflux from cardiac muscle on temperature and external ion composition. *J. Physiol.* **195**: 451-470.
- S.-Rozsa, K. 1984.** The pharmacology of molluscan neurons. *Prog. Neurobiol.* **23**: 79-150.
- S.-Rozsa, K., and T. Kiss. 1976.** Role of cyclic nucleotides in the effect of transmitters on the heart of *Helix pomatia* L. *Comp. Biochem. Physiol. C* **53**: 13-16.
- Sanger, J. W. 1979.** Cardiac fine structure in selected arthropods and molluscs. *Am. Zool.* **19**: 9-27.
- Sawada, M., M. Ichinose, I. Ito, T. Maeno, and D. J. McAdoo. 1984.** Effects of 5-hydroxytryptamine on membrane potential, contractility, accumulation of cAMP, and Ca²⁺ movements in anterior aorta and ventricle of *Aplysia*. *J. Neurophysiol.* **51**: 361-374.
- Smith, P. J. S., K. Hammar, D. M. Porterfield, R. H. Sanger, and J. R. Trimarchi. 1999.** A self-referencing, non-invasive, ion selective electrode for single cell detection of trans-plasma membrane calcium flux. *Microsc. Res. Tech.* **46**: 398-417.
- Suzuki, M., K. Muraki, Y. Imaizumi, and M. Watanabe. 1992.** Cyclopiazonic acid, an inhibitor of the sarcoplasmic reticulum Ca²⁺-pump, reduces Ca²⁺-dependent K⁺ currents in guinea-pig smooth muscle cells. *Br. J. Pharmacol.* **107**: 134-140.
- Vaghy, P. L., K. Itagaki, K. Miwa, E. McKenna, and A. Schwartz. 1988.** Mechanism of action of calcium modulator drugs. Pp. 176-186 in *Calcium Antagonists: Pharmacology and Clinical Research*, Vol. 522. P. M. Vanhoutte, R. Paoletti, and S. Govoni, eds. New York Academy of Sciences, New York.
- Yeoman, M. S., B. L. Brezden, and P. R. Benjamin. 1999.** LVA and HVA Ca²⁺ currents in ventricular muscle cells of the *Lymnaea* heart. *J. Neurophysiol.* **82**: 2428-2440.
- Zahradnik, I., and J. Zachar. 1983.** Inhibitory effect of verapamil upon calcium and potassium currents in crayfish muscle membrane. *Gen. Physiol. Biophys.* **2**: 181-192.